SUPPORTING INFORMATION

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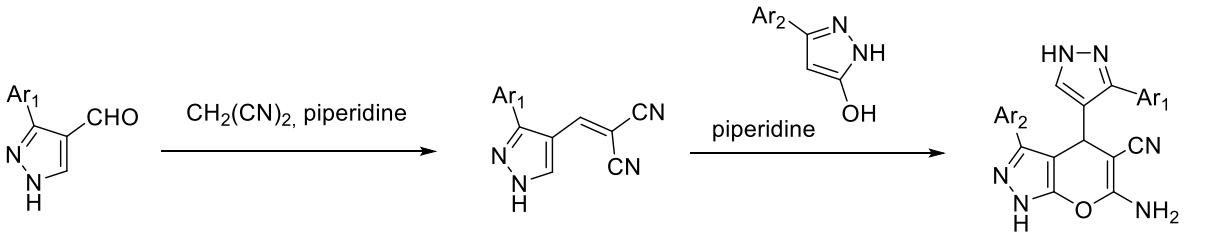
I. Synthesis of the inhibitor HAM-1

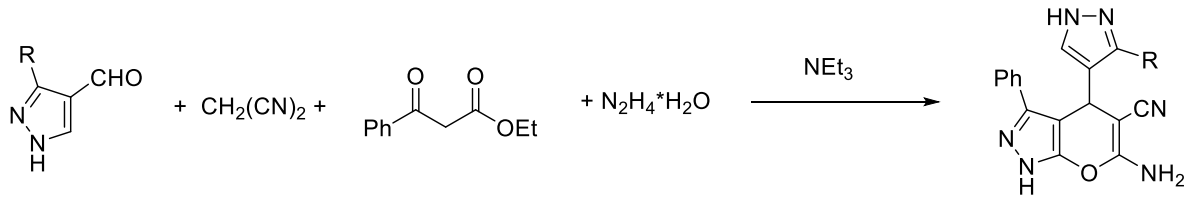
All HAM-1 analogs were prepared according to one of the following methods (Scheme 1).

Method 1



Ar_1, Ar_2 = C_6H_5, 4-FC_6H_4

Method 2

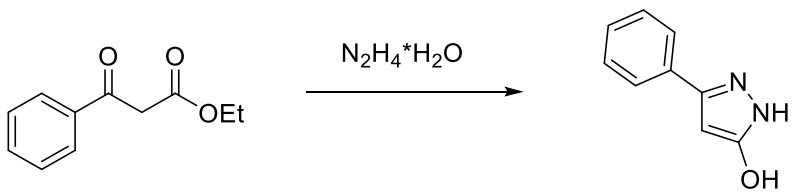


R = Me, Ph

General remarks

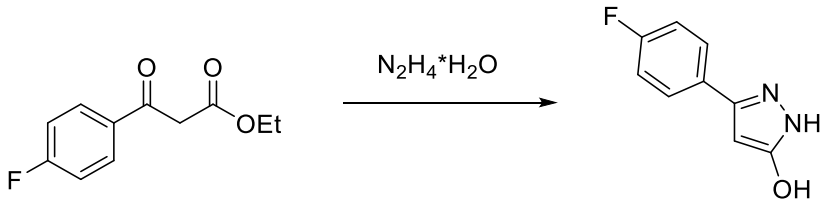
All reactions were carried out under argon in oven-dried glassware unless noted otherwise. All chemicals were of reagent grade or better, and used without further purification. Chemicals and solvents were purchased from Sigma Aldrich (St. Louis, USA). Solvents for chromatography and workup purposes were generally of reagent grade. In all reactions, temperatures were measured externally. ^{1}H NMR and ^{13}C spectra of small molecules were recorded on Bruker instruments (250MHz, 360 MHz or 500 MHz, Bruker) and referenced to the residual proton signal of the deuterated solvent. Carbon samples were reference externally against the residual ^{13}C signal of $CDCl_3$. HR-MS-ESI spectra were recorded with a Thermo Scientific LTQ FT.

5-Phenyl-3-hydroxy-2*H*-pyrazole



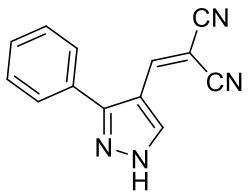
Ethyl 3-phenyl-3-oxopropanoate (3.8 g, 20 mmol) was dissolved in EtOH (50 mL). Acetic acid (5 mL) and hydrazine hydrate (1.1 mL, 1.1 g, 22 mmol) were added to this solution and the reaction mixture was stirred at reflux for 1 h. The volatiles were removed under reduced pressure and the residue was purified by column chromatography (hexane: EtOAc = 1 : 1 to 0 : 1) yielding 1.9 g (61%) of the desired product. – ^{1}H NMR (500 MHz, *DMSO-d$_6$*): 11.77 – 9.72 (br. m, 1 H), 7.68 (d, *J* = 7.5 Hz, 2 H), 7.40 (t, *J* = 7.5 Hz, 2 H), 7.30 (t, *J* = 7.5 Hz, 1 H) 5.91 (s, 1 H). ^{13}C NMR (125 MHz, *DMSO-d$_6$*): 161.51, 143.78, 130.95, 129.26, 128.24, 125.18, 87.33. –HRMS (ESI) calcd. for $C_9H_9N_2O$ $[M+H]^+$ 161.0715, found 161.0709.

5-(4-Fluorophenyl)-3-hydroxy-2*H*-pyrazole



Ethyl 3-(4-fluorophenyl)-3-oxopropanoate (500 mg, 2.4 mmol) was dissolved in EtOH (10 mL). Acetic acid (0.5 mL) and hydrazine hydrate (130 µL, 130 mg, 2.6 mmol) were added to this solution and the reaction mixture was stirred at reflux for 1 h. The volatiles were removed under reduced pressure and the residue was purified by column chromatography (hexane: EtOAc = 1 : 1 to 0 : 1) yielding 240 mg (56%) of the desired product. – ^{1}H NMR (400 MHz, *DMSO-d$_6$*): 12.23–11.75 (br. m, 1 H), 10.06–9.26 (br. m, 1 H), 7.70 (dd, *J* = 8.9, 5.4 Hz, 2 H), 7.23 (t, *J* = 8.9 Hz, 2 H), 5.86 (s, 1 H). –HRMS (ESI) calcd. for $C_9H_8FN_2O$ $[M+H]^+$ 179.0621, found 179.0614.

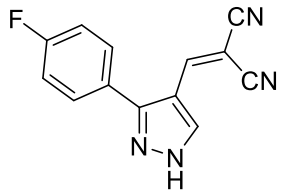
4-(2,2-Dicyanovinyl)-3-phenyl-1*H*-pyrazole



3-Phenyl-1H-pyrazol-4-carbaldehyde (1.0 g, 5.81 mmol) and propanedinitrile (384 mg, 5.81 mmol) were dissolved in EtOH (25 mL) and 5 drops of piperidine were added. The solution was stirred at reflux for 2 h. The volatiles were removed under reduced pressure and the residue was purified by column chromatography (hexane: EtOAc = 2 : 1 to 1: 1) yielding 1.2

g (95%) of the desired product. R_f = 0.68 (hexane: EtOAc = 1 : 1). – ^{1}H NMR (500 MHz, *DMSO-d$_6$*): 14.36 – 14.03 (br. m, 1 H), 8.82 – 8.30 (br.m, 1 H), 8.05 (s, 1 H), 7.66 – 7.45 (m, 5 H). – ^{13}C NMR (126 MHz, *DMSO-d$_6$*): 153.10, 148.00, 139.67, 130.58, 129.56 (2xC), 126.92, 115.11, 114.94, 112.98, 76.50. –HRMS (ESI) calcd. for $C_{13}H_9N_4$ $[M+H]^+$ 221.0827, found 221.0820.

4-(2,2-Dicyanovinyl)-3-(4-fluorophenyl)-1*H*-pyrazole



3-(4-Fluorophenyl)pyrazol-4-carbaldehyde (500 mg, 2.63 mmol) and propanedinitrile (174 mg, 2.63 mmol) were dissolved in EtOH (10 mL) and 5 drops of piperidine were added. The solution was stirred at reflux for 2 h. The volatiles were removed under reduced pressure and the residue was purified by column chromatography (hexane: EtOAc) yielding 530 mg (85%) of the desired product. – ^{1}H NMR (400 MHz, *DMSO-d$_6$*): 14.35 – 14.00 (br. m, 1 H), 8.85 – 8.29 (br.m, 1 H), 8.05 (s, 1 H), 7.71 – 7.57 (m, 2 H), 7.47 – 7.30 (m, 2 H). –HRMS (ESI) calcd. for $C_{13}H_8FN_4$ $[M+H]^+$ 239.0733, found 239.0726.

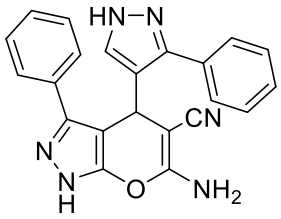
General procedure 1 (GP1) for the synthesis of 6-amino-4-(3-aryl-1*H*-pyrazol-4-yl)-3-aryl-2*H*,4*H*-pyrano[2,3-c]pyrazol-5-carbonitriles

To a solution of 5-aryl-3-hydroxypyrazol (1.35 mmol) in EtOH (10 mL) was added 4-(2,2-dicyanovinyl)-3-arylpyrazol (1.35 mmol) followed by 5 drops of piperidine and the reaction mixture was heated under reflux for 1 h. The volatiles were removed under reduced pressure and the residue was separated by column chromatography (hexane : EtOAc) yielding the desired product.

General procedure 2 (GP2) for the synthesis of 6-amino-4-(3-aryl-1*H*-pyrazol-4-yl)-3-aryl-2*H*,4*H*-pyrano[2,3-c]pyrazol-5-carbonitriles

Propanedinitrile, $N_2H_4 \cdot H_2O$ and ethyl 3-aryl-3-oxopropanoate were simultaneously added to the solution of 3-aryl-1*H*-pyrazol-4-carboxaldehyde in EtOH. Subsequently Et_3N was added to the reaction mixture and it was stirred at reflux for 45 min. The reaction mixture was allowed to cool down to r. t. The volatiles were removed under reduced pressure. Purification by column chromatography yielded the desired product.

6-Amino-4-(3-phenyl-1*H*-pyrazol-4-yl)-3-phenyl-2*H*,4*H*-pyrano[2,3-c]pyrazol-5-carbonitrile (HAM-1a)

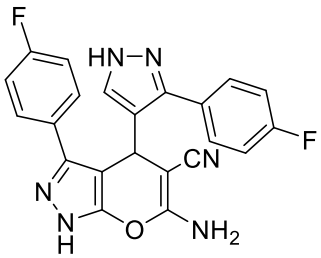


Method 1: 6-Amino-4-(3-phenyl-1H-pyrazol-4-yl)-3-phenyl-2H,4H-pyrano[2,3-c]pyrazol-5-carbonitrile (HAM-1a) was prepared according to GP1 from 5-phenyl-3-hydroxypyrazol (475 mg, 3.0 mmol), 4-(2,2-dicyanovinyl)-3-phenylpyrazol (653 mg, 3.0 mmol) and 9 drops of piperidine in EtOH (22 mL). The purification of the crude product by column chromatography (hexane : EtOAc = 1 : 1) yielded 449 mg (40%) of a desired product.

Method 2: 6-Amino-4-(3-phenyl-1H-pyrazol-4-yl)-3-phenyl-2H,4H-pyrano[2,3-c]pyrazol-5-carbonitrile (HAM-1a) was prepared according to GP2 from propanedinitrile (0.33 g, 5.0 mmol), $N_2H_4 \cdot H_2O$ (0.27 mL, 5.5 mmol), 3-phenyl-1*H*-pyrazol-4-carboxaldehyde (0.88 g, 5.0 mmol), ethyl 3-phenyl-3-oxopropanoate (1.10 g, 5.5 mmol) and Et_3N (0.14 mL, 1 mmol) in EtOH (20 mL). The purification by column chromatography (hexane : EtOAc = 1 : 1) yielded 1.1 g (58%) of a desired product. The product was additionally purified by recrystallization from the mixture of CH_2Cl_2 and EtOH.

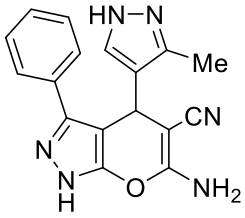
R_f = 0.21 (hexane: EtOAc = 1 : 1). – ^{1}H NMR (500 MHz, *DMSO-d*$_6$): 12.81 – 12.65 (br. s, 2 H), 7.57 – 7.31 (m, 6 H), 7.24 – 7.18 (m, 1 H), 7.18 – 7.06 (m, 4H), 6.92 (s, 2 H), 5.03 (s, 1 H). – ^{13}C NMR (126 MHz, *DMSO-d*$_6$): 160.58, 155.76, 138.04, 129.02, 128.80 (2x2C), 128.60 (2xC), 128.53, 126.50 (2x2C), 121.71, 98.74, 56.50, 26.81. –HRMS (ESI) calcd. for $C_{22}H_{17}N_6O$ $[M+H]^+$ 381.1464, found 381.1458.

6-Amino-4-(3-(4-fluorophenyl)-1*H*-pyrazol-4-yl)-3-(4-fluorophenyl)-2*H*,4*H*-pyrano[2,3-c]pyrazol-5-carbonitrile



6-Amino-4-(3-(4-fluorophenyl)-1H-pyrazol-4-yl)-3-(4-fluorophenyl)-2H,4H-pyrano[2,3-c]pyrazol-5-carbonitrile (HAM-1) was prepared according to GP1 from 5-(4-fluorophenyl)-3-hydroxypyrazol (240 mg, 1.35 mmol), 4-(2,2-dicyanovinyl)-3-(4-fluorophenyl)pyrazol (321 mg, 1.35 mmol) and 5 drops of piperidine in EtOH (10 mL). The purification of the crude product by column chromatography (hexane : EtOAc = 1 : 1 to 0 : 1) yielded 110 mg (20%) of the desired product as a mixture *ca*. 55 : 45 of two rotamers. – ^{1}H NMR (500 MHz, *DMSO-d*$_6$): 12.87 – 12.60 (m, 2 H), 7.54 (s, 0.55 H), 7.42 – 7.27 (m, 2.45 H), 7.26 – 7.11 (m, 4 H), 7.01 (s, 2 H), 6.95–6.81 (m, 2 H), 5.06 (s, 0.55 H), 4.99 (s, 0.45 H). – ^{13}C NMR (126 MHz, *DMSO-d*$_6$): 170.86, 163.15 (d, $J_{C\text{-}F}$ = 66 Hz), 163.09, 161.23 (d, $J_{C\text{-}F}$ = 66 Hz), 161.13, 160.54, 160.41, 155.69, 148.37, 139.92, 138.57, 137.45, 131.19, 130.55 (d, $J_{C\text{-}F}$ = 8 Hz), 129.57, 128.97, 126.68, 125.65 (d, $J_{C\text{-}F}$ = 3 Hz), 121.60, 121.34, 121.27, 116.13, 115.97, 115.75 (d, $J_{C\text{-}F}$ = 11 Hz), 115.36, 115.19, 98.53, 98.37, 58.69, 58.37, 27.00, 26.71. –HRMS (ESI) calcd. for $C_{22}H_{15}F_2N_6O$ $[M+H]^+$ 417.1275, found 417.1276.

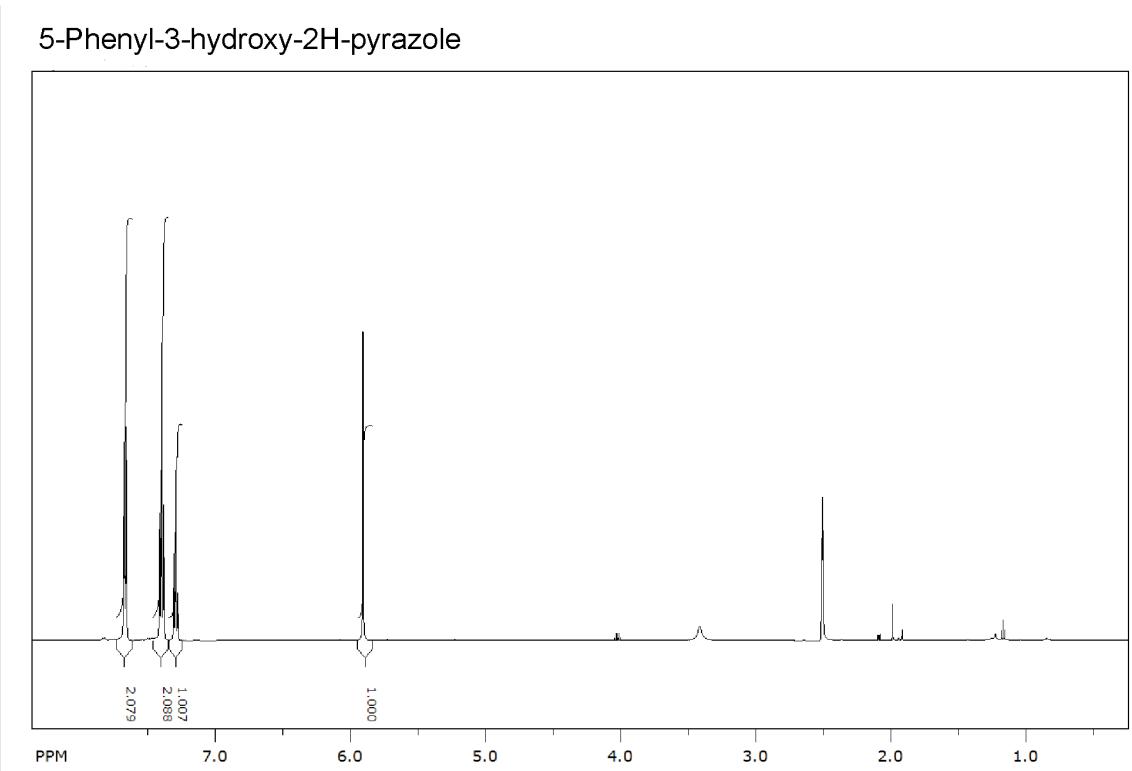
6-Amino-4-(3-methyl-1*H*-pyrazol-4-yl)-3-phenyl-2*H*,4*H*-pyrano[2,3-c]pyrazol-5-carbonitrile (HAM-1c)



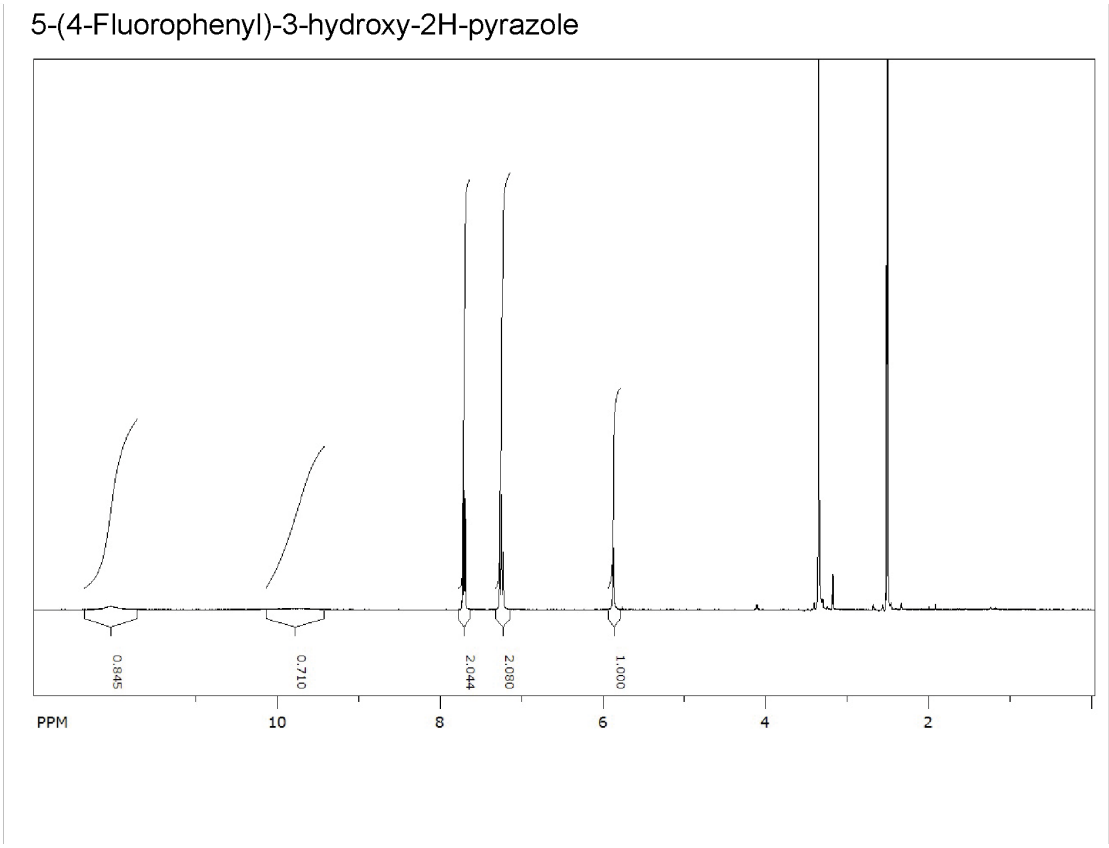
6-Amino-4-(3-methyl-1H-pyrazol-4-yl)-3-phenyl-2H,4H-pyrano[2,3-c]pyrazol-5-carbonitrile (HAM-1c) was prepared according to GP2 from propanedinitrile (0.33 g, 5.0 mmol), $N_2H_4 \cdot H_2O$ (0.27 mL, 5.5 mmol), 3-methyl-1*H*-pyrazol-4-carboxaldehyde (0.55 g, 5.0 mmol), ethyl 3-phenyl-3-oxopropanoate (1.10 g, 5.5 mmol) and Et_3N (0.14 mL, 1 mmol) in EtOH (30 mL). The purification by column chromatography (hexane : EtOAc) yielded 0.72 g (45%) of a desired product. – ^{1}H NMR (360 MHz, *DMSO-d*$_6$): 12.83 (s, 1 H), 12.31 – 12.02 (br. s, 1 H), 7.50 – 7.44 (m, 2 H), 7.38 – 7.14 (m, 4 H), 6.81 (s, 2 H), 4.98 (s, 1 H), 1.98 (s, 3 H). – ^{13}C NMR (90MHz, *DMSO-d*$_6$): 160.28, 156.20, 138.21, 129.28, 128.95, 128.64, 126.67, 121.34, 120.69, 97.91, 58.73, 49.07, 27.34. –HRMS (ESI) calcd. for $C_{17}H_{15}N_6O$ $[M+H]^+$ 319.1307, found 319.1304.

^{1}H-NMR spectra of the synthesized compounds:

A



B



II. Supplementary Figure S1

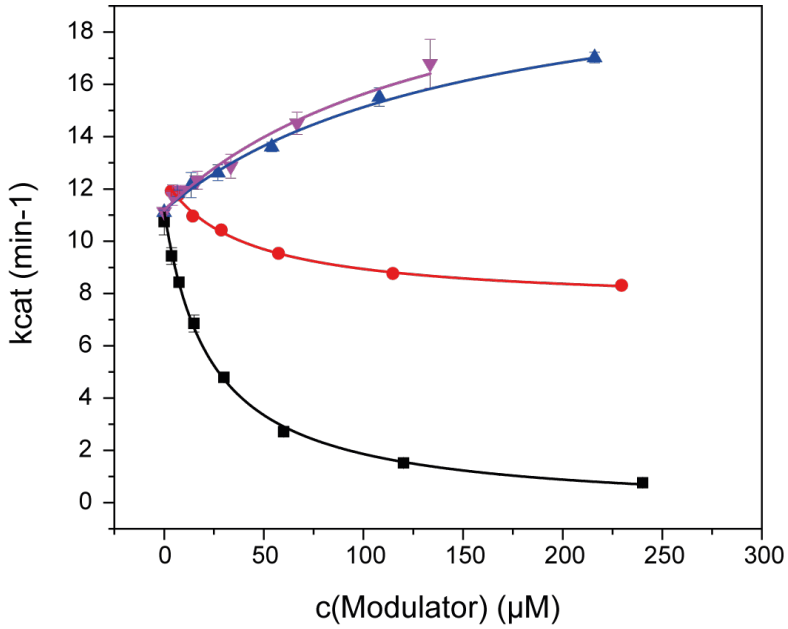


Figure S1: Fits of titrations of the modulators to Hsp90/Aha1 in the regenerative ATPase assay. The Michaelis Menten model was applied to obtain apparent affinities (kDapp) for the modulators. Black: HAM-1, kDapp 23.5 ± 1.7 µM. Red: HAM-2, kDapp 40.3 ± 5.2 µM. Blue: HAM-4 kDapp 163 ± 42 µM. Lilac: HAM-5 kDapp 145 ± 70 µM.

III. Supplementary Figure S2

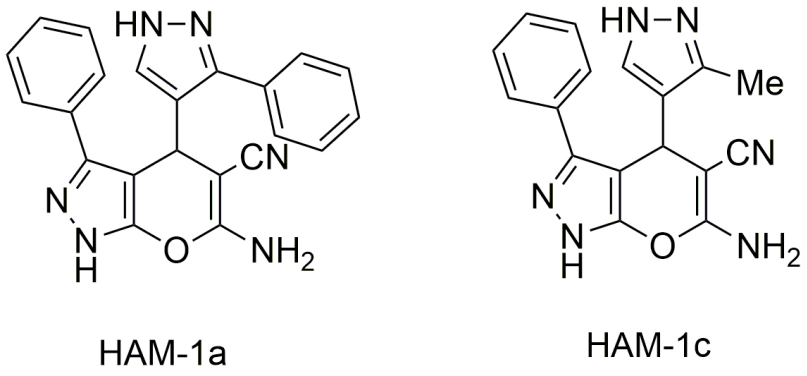


Figure S2: Derivatives of HAM-1. Structures of the HAM-1 derivatives generated in this work.

IV. Supplementary Figure S3

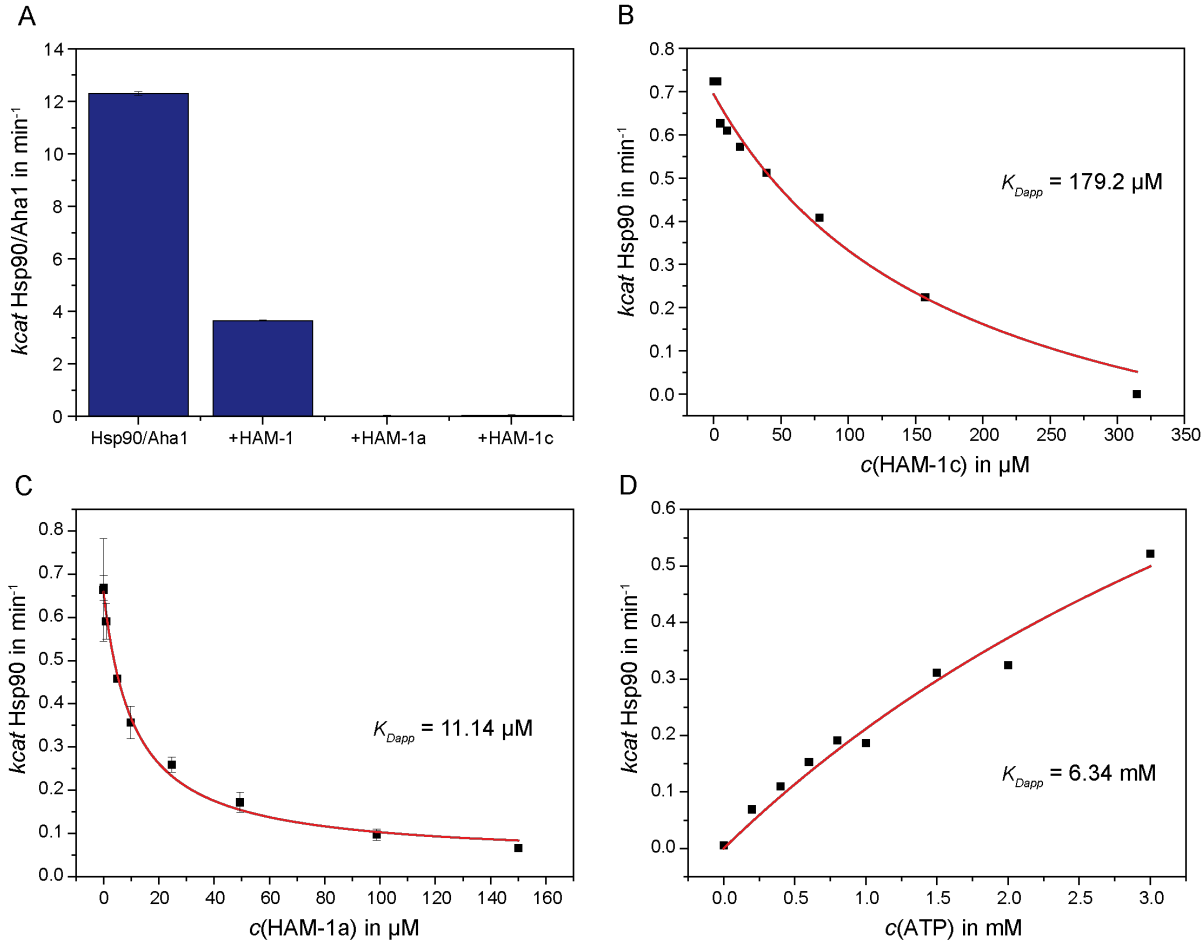


Figure S3: HAM-1 derivative effects on the Hsp90 ATPase activity. A) Effects of HAM-1 and its derivatives on the stimulatory effect of Aha1 (20 µM) on Hsp90 (1µM). HAM-1a and 1c completely inhibit the Hsp90 ATPase activity. B) Titration of HAM-1c to 3 µM Hsp90. C) Titration of HAM-1a to 3 µM Hsp90. D) Effect of HAM-1a on the ATP affinity of Hsp90. 0-3 mM ATP were titrated to 3 µM Hsp90 in the presence of 100 µM HAM-1a to determine K_{Dapp}.

V. Supplementary Figure S4

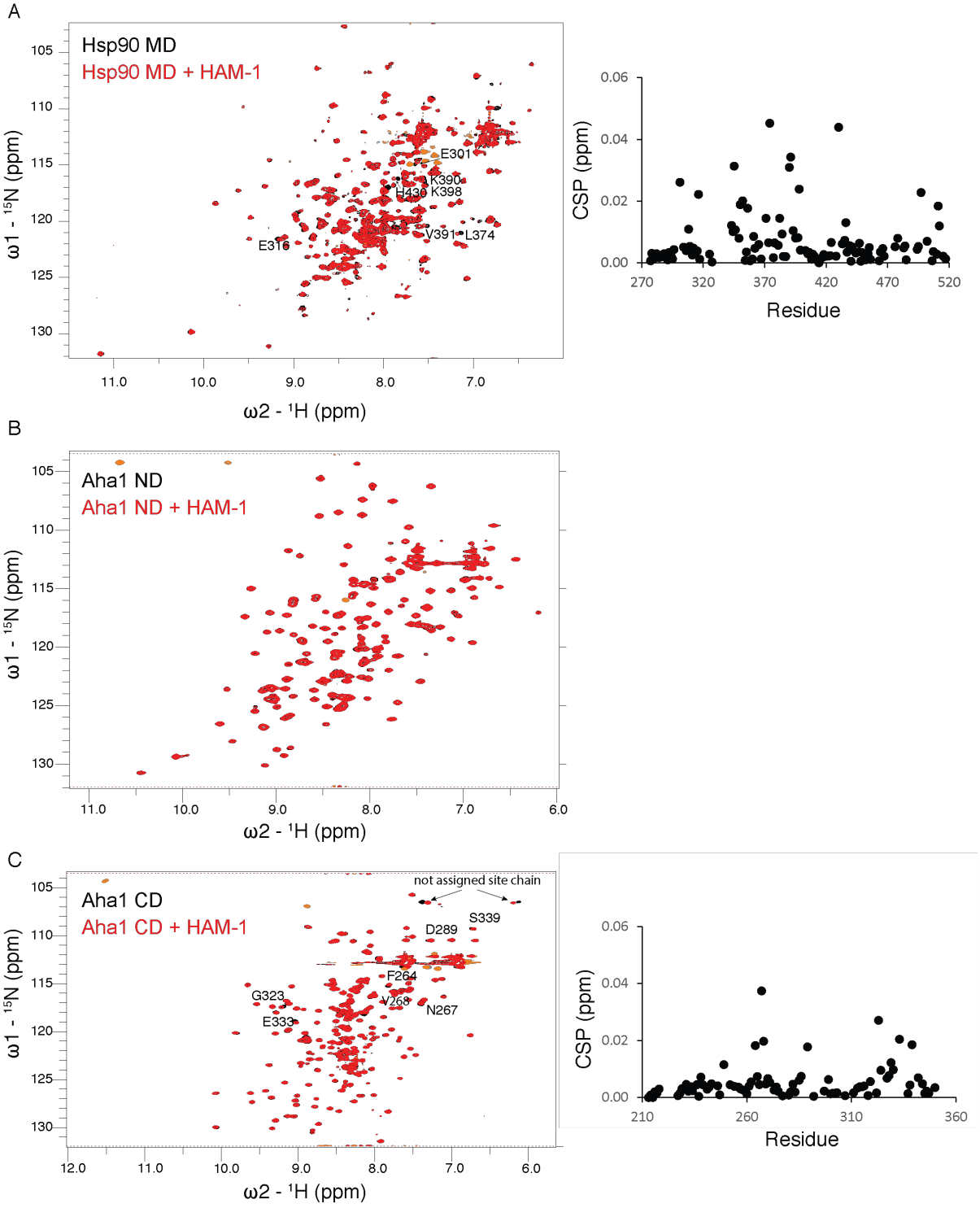


Figure S4: NMR analysis of the HAM-1 interaction with the Hsp90 MD and Aha1 domains. Shown are superimpositions of $^{1}H,^{15}N$ correlation spectra of ^{15}N-labeled Hsp90 MD (A), Aha1 ND (B) and Aha1 CD (C) in the absence (black) and presence (red) of HAM-1. Residues experiencing significant chemical shift changes are annotated in the spectra. On the right panel in A and C the chemical shift perturbation extracted from the spectra per residue is plotted. For the Aha1 ND NMR backbone chemical shift assignments are not available.

VI. Supplementary Figure S5

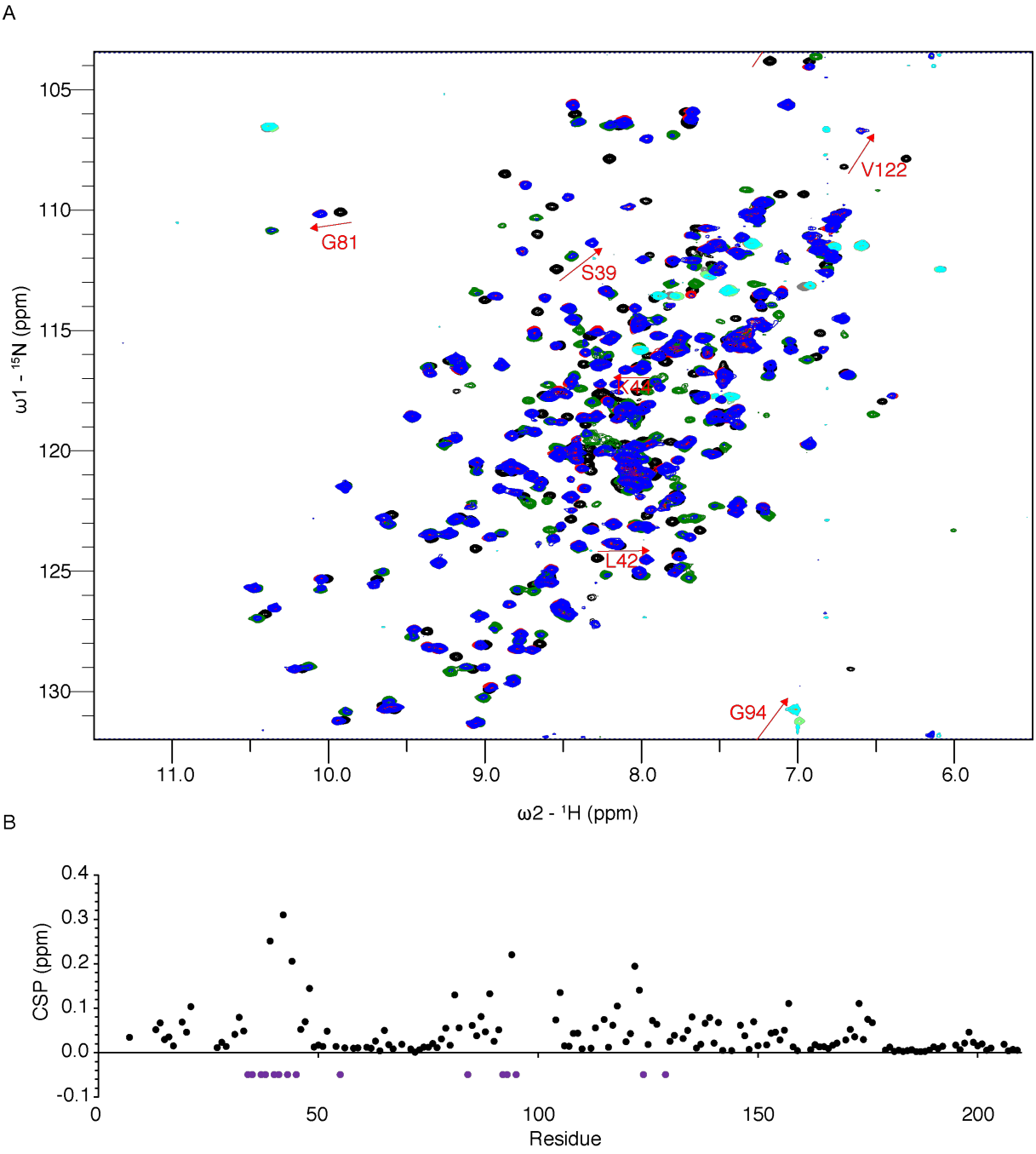


Figure S5: NMR analysis of the Hsp90 NTD/HAM-1 interaction. A) $^{1}H,^{15}N$ HSQC spectrum of the Hsp90 NTD (black) superimposed with spectra of the complex with HAM-1 (red), the ATP bound form (green) and the complex with HAM-1 in the presence of ATP (blue). Red arrows highlight large changes in chemical shift upon HAM-1 binding. B) Chemical shift perturbations (CSPs) between the free Hsp90 NTD and the HAM-1 complex are plotted. Purple data points at -0.05 ppm indicate residues with large CSPs that could not be traced.

VII. Supplementary Figure S6

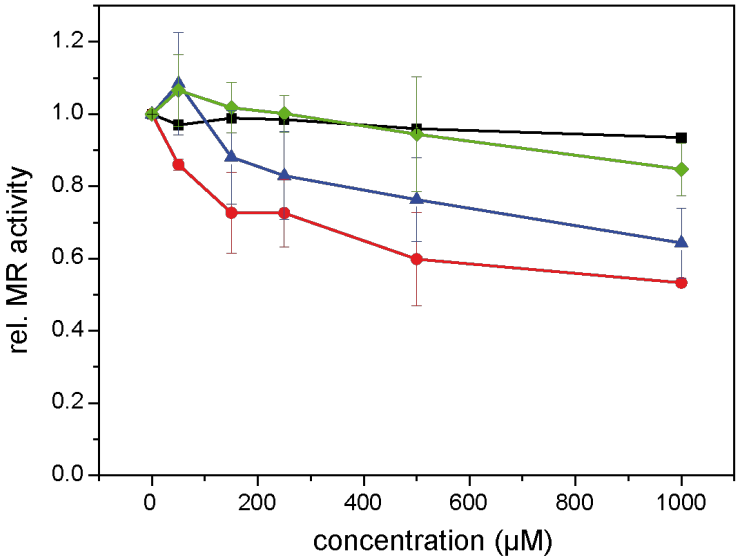


Figure S6: Effects of HAM-1 derivatives on the MR activity in vivo. HAM-1 (unmodified, red) displays the strongest and most specific effect on MR activity in vivo compared to the generated derivatives. HAM-1a (blue), HAM-1c (green), DMSO Control (black).